The Growth Hormone: Insulin-like Growth Factor 1 Axis Is a Mediator of Diet Restriction-induced Inhibition of Mononuclear Cell Leukemia in Fischer Rats

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ABSTRACT

A leukemia cell transplant model and both in situ and in vitro bioassays were used to assess the roles of endogenous factors in mediating diet restriction (DR)-induced inhibition of mononuclear cell leukemia (MNCL) in Fischer 344 rats. DR-treated male rats (n = 35), which were fed 75% of ad libitum (AL) intake of NIH-07 open formula diet, had lower transplanted MNCL incidence (54 versus 77%; P = 0.039) with longer latency (P = 0.015) and decreased severity (P = 0.01) than AL-treated rats 12 weeks after inoculation with MNCL cells. Five-day proliferation rates of cultured MNCL (CRNK-16) cells in diffusion chambers implanted in DR-treated rats were 22% less than in AL-treated rats (P = 0.03), indicating that DR-dependent diffusible factors modulate in situ MNCL cell growth. Secretion of DR-treated rats supported lower in vitro CRNK-16 cell proliferation rates relative to serum from AL-treated rats. Serum levels of both growth hormone (GH) and insulin-like growth factor 1 (IGF-1) were over 50% lower in DR-versus AL-treated rats. An evaluation of the in vitro cell proliferative activity of a panel of purified factors showed that GH and IGF-1, but not 15 other growth factors, stimulated thymidine incorporation in CRNK-16 cells. Infusion of either GH or IGF-1 via osmotic minipumps restored in situ and in vitro CRNK-16 cell proliferation in DR-treated rats up to rates measured in AL-treated rats. Splenocytes from DR-treated rats, relative to AL-treated rats, were more sensitive to mitogen stimulation, displayed increased cell surface expression of receptors for class 1 and 2 major histocompatibility complex molecules, and were more cytotoxic to target tumor cells. Infusion of either GH or IGF-1 in DR-treated rats further enhanced mitogen responsiveness and natural cytotoxicity but reversed the DR-induced increase in major histocompatibility complex receptors. We conclude that DR modulates MNCL progression in Fischer 344 rats through both its influence on MNCL cell proliferation via suppression of the GH:IGF-1 axis and its enhancement of host defenses against tumor cells.

INTRODUCTION

DR is one of the most effective but least understood dietary manipulations for decreasing tumor development in animals. The protective effect of DR against tumorigenesis is well documented and is not limited to a small group of related tumors, to a particular species, nor to a specific mode of tumor induction. In fact, DR inhibits the development of a variety of spontaneous, chemically and physically induced tumors, including leukemia (1–3). However, the mechanism(s) responsible for the tumor inhibitory effects of DR have not been adequately elucidated. Two often-suggested hypotheses to explain this phenomenon include: (a) perturbation of the hormonal milieu of the host by DR resulting in reduced tumor cell proliferation; and (b) DR-induced enhancement of host defenses against tumor cells (1, 2).

Evidence suggesting a possible role for altered pituitary hormone levels in mitigating leukemia cell proliferation comes from studies showing that hypophysectomized rats are resistant to transplanted, virally and chemically induced leukemia (4, 5). DR has been termed a “pseudohypophysectomy” treatment to reflect the decreased levels of GH, prolactin, gonadotropins, and related endogenous factors often observed in DR-treated animals (6). Which, if any, of these hormones mediate the antileukemic effect of DR is not known, although GH is a reasonable candidate since GH injection increases the incidence of radiation-induced lymphoid leukemia in Long-Evans rats (7). In addition, GH and its somatomedin IGF-1 are mitogenic for some in vitro leukemia cell lines (8, 9).

A second hypothesis of how DR inhibits tumors suggests that DR enhances host immune defenses against neoplastic cells. DR may stimulate both the cell-mediated and humoral arms of the immune system, as indicated by proliferative responses of peripheral blood lymphocytes or splenocytes to T- and B-lymphocyte mitogens (10, 11). DR also increases natural cytotoxicity against target tumor cells (12). Mechanisms of DR-induced alterations in immune competence are not completely understood, although reduced GH and IGF-1 levels may be involved since lymphocytes express receptors for GH and IGF-1 (13) and GH plays a regulatory role in certain immune responses (14).

MNCL is a malignant neoplasia which occurs spontaneously at a high rate in aging F344 rats (15). This neoplasia is also known as large granular lymphocytic leukemia since the mononuclear cells involved contain large azurophilic granules characteristic of large granular lymphocytes (16). The incidence of spontaneous MNCL is significantly decreased by corn oil gavage (17), suggesting that this tumor is sensitive to nutritional modulation. The effect of DR on MNCL has not been well characterized, although the incidence of spontaneous MNCL in a lifetime DR study was observed to be lower relative to AL-fed rats (18).

A great deal of interest has developed in the roles of hormones and growth factors as regulators of tumor cell proliferation (1, 19, 20). Most of these factors target specific tissues, while a small number of factors, including the pituitary hormones, adrenal glucocorticoids, insulin, and IGF-1, act on tissues in general to regulate growth and metabolism. Since DR appears to be almost universal with respect to site of tumor inhibition and can modulate the serum levels of numerous endogenous factors (1, 6), we focused on globally active hormones and growth factors as potential mediators of the DR-induced inhibition of MNCL.

In the present study we used a MNCL transplant model, in situ and in vitro MNCL cell proliferation assays, and immune competence assays to address the following questions: (a) does DR retard transplanted MNCL progression? (b) do diet-sensitive endogenous factors, especially the GH:IGF-1 axis, alter MNCL cell proliferation? and (c) does DR alter immune competence?
MATERIALS AND METHODS

Animals. Fifty day-old (160–180-g) male F344 rats (Charles River, Raleigh, NC) were individually housed in polycarbonate cages on hardwood bedding in rooms maintained at 24°C with 12-h light/dark cycles. All rats were given unlimited access to distilled water and received either AL or restricted amounts of NIH-07 open formula diet (Ziegler Brothers Inc., Gardners, PA).

Feed restriction treatments were imposed as follows: daily feed intake of each AL-fed animal was measured and 75% of the mean daily AL intake was administered to diet-restricted animals. This DR regimen exceeded all nutrient requirements as recommended by the National Research Council (21).

Cells and Culture Medium. MNCL cells collected from leukemic F344 rats were used in leukemia cell transplant studies. In brief, malignant mononuclear cells were isolated by density centrifugation from the spleens of three aged F344 rats with spontaneous cases of MNCL. These cells were pooled, frozen, and stored in liquid nitrogen. Previous studies have demonstrated that s.c. injection of MNCL cells (10⁷ cells/rat) into male F344 rats induces leukemia within 60 days that is clinically identical to spontaneous MNCL (22).

An immortalized MNCL cell line (CRNK-16 cells; generously provided by Dr. C. Reynolds, National Cancer Institute) was used for in situ and in vitro cell proliferation assays. CRNK-16 cells are a stable in vitro passaged cell line derived from mononuclear cells isolated from the spleen of a male F344 rat with spontaneous MNCL and established in culture. These cells were maintained in vitro at 37°C and 5% CO₂ in growth medium consisting of RPMI 1640 with 10% heat-inactivated FCS, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 1% antibiotic/antimycotic and 2 × 10⁻⁵ M 2-mercaptoethanol (all from GIBCO/BRL, Gaithersburg, MD). This medium also contained 20% Con A-conditioned medium, made by incubating splenocytes isolated from male F344 rats in the above medium + 10 μg/ml Con A (Sigma Chemical Co., St. Louis, MO). CRNK-16 cells between passage number 30 and 75 were used in these experiments; no further changes in karyotype and 75 were used in these experiments; no further changes in karyotype.

MNCL Transplant Studies. Rats were inoculated (s.c.) in the mid-back with 2 × 10⁶ MNCL cells (0.2 ml in RPMI 1640) following 4 weeks of dietary treatment. Body weights, food intake, and clinical signs of MNCL, including pale eyes, jaundice, and palpable spleen were recorded weekly. Moribund leukemic animals were sacrificed when body weight loss exceeded 20% of peak weight. All surviving animals were sacrificed at 84 days posttransplant. At sacrifice, spleen weights were recorded and sections of spleen and liver were excised and stored in 10% buffered formalin. MNCL was confirmed and staged (15) by histological analysis of paraffin sections of this tissue stained with hematoxylin and eosin. Incidence (percentage of animals in a treatment group which develop MNCL) was compared between treatment groups using a Yates-corrected χ² test. Banding pattern, population doubling time, or ability to induce leukemia when transplanted were observed through 120 passages, attesting to their stability throughout the course of our studies.

CRNK-16 cells are a stable in vitro passaged cell line transplanted were observed through 120 passages, attesting to their stability throughout the course of our studies.

In Situ Proliferation Assay. The in situ proliferation assay is a modification of DC (Millipore Corp., Bedford, MA) procedures described by Carsten (24). A DC consists of one lucite O-ring (13-mm outer diameter) to which 2 polycarbonate filters (0.2-μm pore size; 13-mm diameter) are cemented. DC were filled with 100 μl (10⁶ cells) of a CRNK-16 cell suspension in SFM, which was the same medium described above without serum or Con A-conditioned media. Two DC were implanted (i.p.) in ketamine/xylazine-anesthetized rats. After 5 days the DC were retrieved and incubated (30 min) in 0.05% Pronase (Sigma) in Dulbecco’s PBS (GIBCO/BRL). Chamber contents were quantitatively emptied in 0.9 ml RPMI 1640 and counted with a hemacytometer. Changes in cell morphology were assessed via microscopic analysis of Wright-Giemsa stained cytospin preparations. A proliferation index (ISPI) of in situ cell growth, relative to baseline values determined from an initial sample of 6 DC loaded with CRNK-16 cells at the time of implantation and then immediately harvested and counted, was calculated using the following formula:

\[
\text{ISPI} = \frac{\text{cell counts in DC 5 days postimplant}}{\text{cell counts in DC at baseline}}
\]

The ISPI values were averaged for each treatment group and the means compared using Student’s t test. When more than two treatment groups were compared, one-way analysis of variance was used. When the analysis of variance suggested statistically significant treatment-related effects, Duncan’s multiple comparison procedure was applied, with Duncan’s test performed at \( P = 0.05 \).

In Vitro Proliferation Assay. We adapted a standard in vitro cell proliferation assay, based on tritiated [³H]thymidine incorporation (25), to quantitate CRNK-16 cell proliferation in response to serum from DR- or AL-treated rats or to purified growth factors. CRNK-16 cells maintained in growth medium described above, were transferred to SFM and incubated for 48 h at 37°C. These cells were then plated in 96-well microtiter plates at 1 × 10⁵ cells/well (100 μl volume). Various dilutions of sera from AL- or DR-treated rats (optimal serum concentration, 0.04%, based on preliminary data showing maximal differences between treatments at this concentration) or of purified factors were made in SFM and plated in triplicate and the plates were incubated for 48 h at 37°C. Purified factors tested include rat GH, prolactin, FSH, luteinizing hormone, and adrenocorticotropic hormone (all gifts from Dr. A. Parlow, NIH); rh IGF-1 (gift from Dr. L. Underwood, University of North Carolina, Chapel Hill, NC); rh IL-1α, -1β, -2, -3, -4, and -6; rh TGF-α and -β; rhe leukemia inhibitory factor; rh tumor necrosis factors α and β; platelet-derived growth factor (all from R and D Systems, Minneapolis, MN); rh epidermal growth factor (Collaborative Research, Cambridge, MA); and porcine insulin (GIBCO). All rh factors included in the analysis had reported activity in rats. Following a 24-h pulse with [³H]thymidine (1 μCi/well), the cells were harvested with an automated cell harvester (Cambridge Technology, Inc., Cambridge, MA) onto glass fiber filters, which were combined with liquid scintillation cocktail (Formula 989; Dupont/NEN, Boston, MA) and counted with a liquid scintillation counter (Beckman Instruments Co., Fullerton, CA). An in vitro proliferation index (IVPI) based on radioactivity cpm of test agents relative to SFM controls was calculated as follows:

\[
\text{IVPI} = \frac{\text{cpm of test agent} - \text{background cpm}}{\text{cpm of SFM controls} - \text{background cpm}}
\]

The background value (nonspecific uptake of radioactivity) was determined by lysing the cells growing in 6 SFM control wells with 5% trichloroacetic acid immediately after plating and incubating and harvesting these wells with the rest of the plate. Comparisons of mean IVPI values between treatments were made using Student’s t test. When more than two treatment groups were being compared, one-way analysis of variance was used. When the analysis of variance suggested statistically significant treatment-related effects, Duncan’s multiple comparison procedure was applied, with Duncan’s test performed at \( P = 0.05 \).

Serum Collection and Analysis. Blood was collected into serum separator tubes (Becton-Dickinson, Rutherford, NJ) from the orbital venous plexus under 70% CO₂/30% O₂ anesthesia or from the inferior vena cava after sacrifice with CO₂. On the day of the blood draw, food was administered to the DR-treated animals from 6:00 a.m. until the start of the light cycle (8:00 a.m.). Food was then removed from all the animals for 2 h, and blood was collected between 10:00 and 10:30 a.m. Clotted blood was centrifuged at 1000 × g for 20 min and the serum was stored at −70°C until assayed. Total serum protein concentration was determined using the enhanced BCA Protein Assay (Pierce Chemical Co., Rockford, IL). Serum IGF-1 levels were measured by radioimmunoassay (generously performed by the laboratory of Dr. L. Underwood, University of North Carolina) using purified human plasma-derived IGF-1 as the standard. Serum levels of GH, prolactin, and ACTH were determined by radioimmunoassay using rat hormone standards and antisera (gifts from Dr. A. Parlow, NIDDK Rat Pituitary Hormone Distribution Program, Baltimore, MD). Serum albumin levels were determined by the bromocresol green assay (26). Differences between AL- and DR-treated rats in mean serum concentrations of these factors were evaluated using Student’s t test, with differences considered statistically significant at \( P \leq 0.05 \).

3 C. Reynolds, personal communication.
**Inhibition of Leukemia by Diet Restriction**

**Effect of Diet Restriction on the Progression of Transplanted MNCL in Male F344 Rats.** As shown in Table 1, DR-treated rats, relative to AL-treated rats, had 30% lower incidence of MNCL (54% versus 77%; P = 0.039 from 2-tailed, Yates-corrected χ² test) at 12 weeks following MNCL cell transplant. In addition, those DR-treated rats which developed MNCL took longer to display initial clinical symptoms (P = 0.015) and had lower mean histological severity scores (P < 0.01) and spleen weights (P < 0.01) than AL-treated rats. DR-treated animals consumed 24% less food (P < 0.01) and weighed 21% less (P < 0.01) than AL-fed rats, indicating that our DR regimen was effective. These data demonstrate that DR slows the progression of transplanted MNCL in our model.

**Effect of Diet Restriction on Serum Factors.** Serum from DR-treated rats averaged approximately 30% of the GH (6.9 versus 23.1; P < 0.001) and 44% of the IGF-1 (519 versus 1188 ng/ml; P < 0.001) levels measured in serum from AL-treated rats (Table 2). No differences were observed between serum from DR- and AL-treated rats in prolactin or ACTH levels. Serum total protein and albumin levels were also not statistically different between treatment groups, indicating that DR-treated rats are not protein malnourished. Thus, levels of GH and its somatomedin IGF-1 were considerably decreased by DR while all other factors measured were unaffected by DR.

**Effect of DR on In Situ Cell Proliferation.** In order to assay the proliferation of MNCL cells in the whole animal under different dietary conditions, we utilized implanted diffusion chambers filled with CRNK-16 cells. The mean ISPI, which is the DC cell count 5 days after implant divided by the baseline DC cell count, was significantly lower in DR-treated rats than in AL-treated rats (Table 3; 23.9 versus 30.4; P = 0.03). These data suggest that DR-sensitive endogenous factors, able to diffuse across the 0.2 µm DC membrane, modulate in situ growth of MNCL cells.

**In Vitro Proliferative Activity of Serum from AL- and DR-Treated Rats.** CRNK-16 cells were grown in culture medium containing serum from AL- or DR-treated rats to evaluate the relative effects of these diet treatments on the proliferative activity of serum. As shown in Table 3, serum from DR-treated rats induced less in vitro CRNK-16 cell proliferation than did serum from AL-treated rats (IVPI = 7.9 versus 10.3; P < 0.05). These data suggest that the

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**Table 1. Effect of diet restriction on transplanted MNCL**

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Ad libitum</th>
<th>Diet restricted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence</td>
<td>77%</td>
<td>54%</td>
</tr>
<tr>
<td>Latency (day of tumor onset)</td>
<td>52 ± 1</td>
<td>57 ± 2b</td>
</tr>
<tr>
<td>Severity (histological stage)</td>
<td>2.6 ± 0.3</td>
<td>1.7 ± 0.3a</td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>153 ± 1</td>
<td>107 ± 3b</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>377 ± 10</td>
<td>298 ± 7b</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>20.1 ± 0.5</td>
<td>15.3 ± 1b</td>
</tr>
</tbody>
</table>

*Ad libitum-fed or diet restricted rat (n = 35/group) were inoculated with 2 x 10⁵ MNCL cells obtained from an in vivo serially passaged cell line (20). Treatments were continued and rats were observed for clinical signs of MNCL, including weight loss, palor effects of these diet treatments on the proliferative activity of serum. Duncan's multiple comparison procedure was used when the analysis of variance suggested statistically significant treatment-related effects, with Duncan's test performed at P = 0.05.*
Table 2 Effect of diet restriction on serum factors

<table>
<thead>
<tr>
<th>Serum factor</th>
<th>Serum concentration</th>
<th>Diet restricted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth hormone (ng/ml)</td>
<td>23.1 ± 3.4</td>
<td>0.9 ± 1.2*</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>1188 ± 21</td>
<td>519 ± 14*</td>
</tr>
<tr>
<td>Prolactin (ng/ml)</td>
<td>20.2 ± 1.7</td>
<td>21.5 ± 1.5</td>
</tr>
<tr>
<td>ACTH (ng/ml)</td>
<td>597 ± 22.8</td>
<td>616 ± 146</td>
</tr>
<tr>
<td>Albumin (ng/ml)</td>
<td>5.2 ± 0.1</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>Total protein (mg/ml)</td>
<td>83.0 ± 3.2</td>
<td>76.0 ± 4.3</td>
</tr>
</tbody>
</table>

a Rats were fed ad libitum or were diet restricted for 4 weeks and blood was drawn prior to inoculation with MNCL cells. Data are expressed as mean ± SE (n = 20/treatment).

b P < 0.05.

d Recombinant human.

Table 3 Effect of diet restriction on MNCL cell proliferation in situ and in vitro

<table>
<thead>
<tr>
<th>Proliferation index</th>
<th>Ad libitum</th>
<th>Diet restricted</th>
</tr>
</thead>
<tbody>
<tr>
<td>In situ</td>
<td>30.4 ± 2.4</td>
<td>23.9 ± 1.7*</td>
</tr>
<tr>
<td>In vitro</td>
<td>10.2 ± 0.4</td>
<td>7.8 ± 1.0*</td>
</tr>
</tbody>
</table>

a Cultured MNCL cells (CRNK-16 cells) were incubated in serum-free medium for 48 h and then added to 96-well microtiter plates (1 x 10^5 cells/well) and incubated for 48 h in serum-free medium with and without serum from ad libitum or diet restricted rats (serum concentrations tested, 0.01–10%; data shown, 0.04%). The cells were pulsed with [3H]thymidine (1 ¿iCi/well) for 24 h, DNA was collected onto glass fiber filters, and radioactivity was counted with a liquid scintillation counter. Data are expressed as mean ± SE (n = 20).

b For definitions, see text.

c P < 0.05.

d In vitro proliferation index = [3H]Thymidine incorporation induced by serum added to serum-free medium

Thymidine incorporation induced by serum-free medium alone

Serum concentration, 0.04%.

Table 4 Proliferative activity of purified factors in vitro

<table>
<thead>
<tr>
<th>Factor (concentration)</th>
<th>In vitro proliferation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin-2* (500 U/ml)</td>
<td>2.5 ± 0.1*</td>
</tr>
<tr>
<td>Transforming growth factor ß* (10 ng/ml)</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>Growth hormone* (800 ng/ml)</td>
<td>2.2 ± 0.03*</td>
</tr>
<tr>
<td>Insulin-like growth factor ß* (10 ng/ml)</td>
<td>1.8 ± 0.01*</td>
</tr>
<tr>
<td>Insulin* (1 µg/ml)</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Follicle-stimulating hormone* (6.25 ng/ml)</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>Prolactin* (2 µg/ml)</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>Adrenocorticotropic hormone* (250 ng/ml)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Luteinizing hormone* (500 ng/ml)</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Interleukin 1α (pg/ml)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Interleukin 1β (10 pg/ml)</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Interleukin 3 (1 ng/ml)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Interleukin 4 (10 ng/ml)</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Interleukin 6 (1 ng/ml)</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Tumor necrosis factor α* (10 pg/ml)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Tumor necrosis factor ß* (100 pg/ml)</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Leukemia inhibitory factor ß* (10 ng/ml)</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>Platelet-derived growth factor* (100 pg/ml)</td>
<td>1.0 ± 0.04</td>
</tr>
<tr>
<td>Transforming growth factor α* (1 ng/ml)</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>Epidermal growth factor* (2.5 ng/ml)</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

a Each factor was added to SFM over a 4-fold range of concentration and tested for in vitro index.

b Recombinant human.

c Different from 1.0 (value for SFM alone) at P < 0.05.

d Recombinant human.

e Recombinant bovine.

DR-sensitive endogenous factors responsible for the modulation of MNCL cell growth are serum borne.

In Vitro Proliferative Activity of Purified Factors. We compared the ability of 20 different protein factors, previously shown to modulate the growth of normal or neoplastic cells (2, 29), to induce DNA synthesis in CRNK-16 cells in vitro. The data presented in Table 4 are for the optimal concentration (maximal differences between treatment groups) of the dose-response curves analyzed for each factor, expressed as the mean ± SE (n = 4/treatment).

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Table 5 Effect of growth hormone or IGF-1 infusion in diet-restricted rats on MNCL cell proliferation in situ and serum-stimulated proliferation in vitro

Data are expressed as mean in situ and in vitro proliferation indices ± SE (n = 15/group for growth hormone infusion study; 8/group for IGF-1 infusion study). For definition of in situ proliferation index, see text.

\[
\text{In vitro proliferation index} = \frac{[^{3}H]\text{Thymidine incorporation induced by serum added to serum-free medium (serum concentration } = 0.04\%)}{[^{3}H]\text{Thymidine incorporation induced by serum-free medium alone}}
\]

Test agent infused growth hormone (6.25 µg/h x 5 days) or IGF-1 (10 µg/h x 5 days) as indicated by column heading. ND, not done. Values within columns with different superscripts are statistically different at \( P < 0.05 \).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth hormone</th>
<th>IGF-1</th>
</tr>
</thead>
</table>
| Ad libitum | \begin{align*} &\text{GH} \\
&\text{IGF-1} \end{align*} | \begin{align*} &\text{GH} \\
&\text{IGF-1} \end{align*} |
| + Saline  | 37.9 ± 3\(^a\) | 21.0 ± 2\(^a\) | 7.1 ± 0.3\(^a\) | 3.1 ± 0.3\(^a\) | 2.0 ± 0.3\(^a\) | 1.7 ± 0.3\(^a\) |
| + Test Agent | 56.0 ± 6\(^b\) | 30.7 ± 3\(^b\) | 9.5 ± 0.3\(^b\) | 5.9 ± 0.4\(^b\) | 3.3 ± 0.3\(^b\) | 2.5 ± 0.3\(^b\) |
| Diet restricted | \begin{align*} &\text{GH} \\
&\text{IGF-1} \end{align*} | \begin{align*} &\text{GH} \\
&\text{IGF-1} \end{align*} |
| + Saline  | 36.2 ± 1.0\(^a\) | 27.9 ± 1.6\(^a\) | 3.4 ± 0.3\(^a\) | 36.8 ± 4.5\(^a\) | 2.2 ± 0.1\(^a\) | 1.47 ± 0.1\(^a\) |
| + Test Agent | 55.0 ± 1.2\(^b\) | 27.9 ± 1.6\(^b\) | 3.4 ± 0.3\(^b\) | 36.8 ± 4.5\(^b\) | 2.2 ± 0.1\(^b\) | 1.47 ± 0.1\(^b\) |

The observed decrease in transplanted MNCL incidence and severity in male F344 rats by DR is in agreement with a previous report of decreased transplanted leukemia in DR-treated mice (30). IGF-1 infusion in DR-treated rats, but not AL-treated rats, increased the percentage of cells staining for both W3/25 and OX8. DR-treated rats infused with saline, relative to AL-treated rats infused with saline, demonstrated greater fluorescence intensity for both W3/25 and OX8, and IGF-1 infusion returned the mean fluorescence intensity values in DR-treated rats to AL-treated levels (Table 8). IGF-1 infusion had no effect in AL-treated rats. The effect of GH on T-cell subsets was not evaluated. Thus, DR induces a reduction in the percentage of splenic T-helper and T-cytotoxic/suppressor cells, although their relative percentages are unchanged, and increases the expression per cell of the major histocompatibility complex molecules against which the W3/25 and OX-8 antibodies are directed.

DISCUSSION

The observed decrease in transplanted MNCL incidence and severity in male F344 rats by DR is in agreement with a previous report of decreased transplanted leukemia in DR-treated mice (30). It is also consistent with inhibited development of radiation-induced leukemia in mice (31, 32), although the severity of the diet restriction regimen used in those mice studies may have resulted in malnutrition in DR animals, complicating the interpretation of their data. The only previous report of the effect of DR on MNCL in male F344 rats was a lifetime mortality study which showed reduced (by approximately 20%) occurrence of spontaneous MNCL in DR-treated rats relative to AL-treated rats (18). Like our DR regimen, the DR diet used in that lifetime study provided adequate nutrition. However, since a cohort of animals was followed throughout their lives, the findings from that study were based on small numbers of spontaneous MNCL cases and
were subject to intercurrent mortality bias due to alterations in the occurrence of other diseases which increased their likelihood of dying from MNCL. Thus, our findings using the MNCL transplant model confirm the observation from the previously published lifetime study that DR inhibits MNCL and suggest that DR slows the growth of transplanted MNCL cells.

Hyphysectomy inhibits experimentally induced leukemia in rats (4, 5). We therefore measured the level of several pituitary hormones in the blood of AL- and DR-treated rats (Table 3). Our findings that GH and IGF-1, but not prolactin or ACTH, are reduced by the DR regimen used in our studies are consistent with reports that the GH:IGF-1 axis is suppressed by DR (33, 34). The level of DR (75% of AL intake) was chosen to ensure against nutrient deficiencies, since consumption of NIH-07 diet at this level exceeds all known nutrient requirements of the rat (21). In addition, the crude protein concentration of NIH-07 diet (23.5%) is high compared to most other open formula rodent diets (i.e., NIH-31 contains 18% crude protein), and DR-treated rats displayed adequate protein nutrition since serum total protein and albumin levels did not differ between AL- and DR-treated rats. Thus we were able to evaluate the effect of DR without inducing malnutrition, a complicating factor in the interpretation of some previous DR studies which used a more severe DR regimen (1). It should be noted that since we imposed a general dietary restriction, it is not possible to attribute the results to the restriction of a particular nutrient or nonnutrient component.

Since DR inhibits in situ CRNK-16 cell growth via alterations in diffusible factor(s) and induces changes in the hormonal milieu of the host, particularly the GH:IGF-1 axis, we used an in vitro proliferation assay to facilitate the identification of the mediating factor(s). Serum from DR-treated rats had less in vitro proliferative activity than serum from AL-treated rats, indicating that the mediating factors are serum borne. A screen of 20 peptide hormones and growth factors which have been shown to modulate the growth of normal and neoplastic cells (2, 29) revealed that besides IL-2 and TGF-β (positive and negative controls, respectively), only GH, IGF-1, and possibly FSH induced CRNK-16 cells to proliferate. These observations attest to some degree of specificity of the modulating effects of GH and IGF-1 on MNCL cell proliferation. A mitogenic effect of GH (8, 35) and IGF-1 (9, 36, 37) for cultured leukemia cells has previously been observed. The proliferative action of GH in most tissues appears to be dependent on the production of IGF-1, and caloric intake and dietary protein have both been shown to regulate serum GH and IGF-1 levels (38, 39). IGF-1 has been implicated as a mediating factor in numerous cancers (40-43) and may be produced by tumor cells as an autocrine and/or paracrine growth factor in response to GH (44, 45). A relationship between FSH levels and the antitumor effects of DR is not clear, although an interaction with the GH:IGF-1 axis is possible since FSH has been shown to synergize with IGF-1 to stimulate granulosa cell proliferation (46).

It should be noted that this screen involved adding individual hormones and growth factors diluted in SFM to CRNK-16 cells, since studies conducted during the development of the assay indicated that a serum-free system was necessary for adequate assay sensitivity. Some factors may require the presence of other factors normally present in FCS to fully express their proliferative activities (47) and may not have activity in a serum-free system. Thus, our findings that CRNK-16 cell proliferation can be modulated by exogenous GH and IGF-1, along with IL-2, TGF-β, and possibly FSH, must be viewed in the context of serum-free culture conditions.

To determine if replacement of GH in DR-treated animals reverses the DR-induced inhibition of cell proliferation in those animals, we infused GH into DR-treated rats implanted with DC loaded with CRNK-16 cells. GH infusion restored in situ CRNK-16 cell proliferation in DR-treated rats up to levels observed in AL-treated rats. In addition, the in vitro proliferative activities and GH levels in serum from DR-treated rats infused with GH were also increased up to levels observed in AL-treated rats. Thus, GH can play a mediating role in the DR-induced inhibition of MNCL cell proliferation since restoration of GH levels by infusion reverses the suppression of proliferative activity in DR-treated rats.

Since GH infusion increases serum IGF-1 levels (27), we could not determine if our observed effects are mediated by GH alone or in combination with IGF-1. Since GH generally exerts its proliferative effects through IGF-1, we also evaluated the effects of IGF-1 infused into AL-fed and DR-treated rats. IGF-1 infusion had no effect on in situ proliferative activity in AL-fed rats, suggesting that the proliferation of CRNK-16 cells in response to IGF-1 levels in AL-fed rats is maximal and refractory to exogenous IGF-1. However, IGF-1 infusion restored in situ CRNK-16 cell proliferation in DR-treated rats to AL levels and increased the in vitro proliferative activity of serum from DR rats to intermediate levels. These data are consistent with the notion that GH acts in concert with its somatomedin to exert its effects on CRNK-16 cell proliferation. Studies are currently underway in our laboratory to evaluate the expression of GH and IGF-1 receptors, using Western blot analysis, and levels of IGF-1 messenger RNA, using Northern blot analysis, in CRNK-16 cells and the MNCL transplant cell line in an effort to further resolve the roles of GH and IGF-1 in the DR-induced inhibition of MNCL cell proliferation.

Altered immune competence has also been purported as a mediator of DR-induced tumor inhibition, although the effects of DR on immunity have not been well characterized. Our findings suggest that DR enhances host immune competence as determined by the responsiveness of splenocytes to T- and B-cell mitogens and the ability of splenocytes, specifically those with natural killer cell activity, to lyse target tumor cells. Enhanced mitogen responsiveness in DR-treated mice has previously been demonstrated (10, 11) while our finding of increased natural cytotoxic activity in DR animals without inducing tumor specific immunity is consistent with findings in our studies and others (12). One possible explanation for this difference is that the Weindruch study used splenocytes from DR-treated mice which, relative to rat splenocytes, tend to display much less affinity for the Yac-1 target tumor cells used in the assay.
We also evaluated the effect of the GH:IGF-1 axis on immune competence in DR-treated male F344 rats, testing the hypothesis that reduced levels of GH and IGF-1 levels, which play a mediating role in DR-induced suppression of CRNK-16 cell proliferation, may also mediate DR-related changes in immunity. These studies demonstrated that: (a) GH or IGF-1 infusion relative to saline infusion increases mitogen responsiveness and natural cytotoxicity in both AL- and DR-treated rats, consistent with findings from Davila et al. (14) that GH can regulate immune function; and (b) the enhanced mitogen responsiveness and natural cytotoxicity observed in DR-treated rats appears to be independent of reduced GH or IGF-1 levels since restoration of GH or IGF-1 levels further increases these immune parameters in DR-treated rats.

The increased responsiveness of splenocytes to mitogens in DR-treated rats is not due to a shift in T-cell subsets, since no diet-related changes were observed in the ratio of T-helper cells to T-cytotoxic/suppressor cells. This finding is consistent with a report that DR does not shift T-cell subsets in young animals, although that report does suggest that age-related changes in subsets are delayed by DR (48). IGF-1 infusion in DR- but not AL-treated rats increased the percentage of both T-helper and T-cytotoxic/suppressor cells. The effects of IGF-1 on immune parameters are not well characterized.

DR-treated rats infused with saline, relative to AL-treated rats infused with saline, demonstrated higher cell surface expression of the receptors for the major histocompatibility class I and II molecules, as determined by mean fluorescence intensity of staining with the W3/25 and OX-8 antibodies. This is, to our knowledge, the first report of an effect of DR on the expression of these molecules. Up-regulation of these receptors on the cell surface of T-cells may facilitate recognition by, and activation of, T-helper cells and might make T-cell-mediated immune function more efficient in DR-treated rats. Interestingly, IGF-1 infusion in DR-treated rats reversed the mean fluorescence intensity level to AL-treated levels while IGF-1 infusion in AL-treated rats had no apparent effect. The DR-induced alterations in mitogen responsiveness and natural cytotoxicity cannot be explained by reduced GH or IGF-1 levels, suggesting distinct mechanisms for the effects of DR on leukemia cell proliferation and enhanced immune competence. However, our findings on the expression of the receptors for the major histocompatibility Class I and II molecules are consistent with a role of the GH:IGF-1 axis in DR-induced effects on immune competence.

In summary, we used an MNCL transplant model, in situ and in vitro leukemia cell proliferation assays, and immune competence assays to investigate the mechanisms by which DR inhibits MNCL. We conclude that DR modulates MNCL progression in Fischer 344 rats through both its influence on MNCL proliferation via suppression of the GH:IGF-1 axis and its enhancement of host defenses against tumor cells. Since diet restriction has been shown to modulate tumor outcomes in a variety of experimental paradigms, opportunities are widespread for evaluating the roles of GH and IGF-1 in mediating these responses.

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In summary, we used an MNCL transplant model, in situ and in vitro leukemia cell proliferation assays, and immune competence assays to investigate the mechanisms by which DR inhibits MNCL. We conclude that DR modulates MNCL progression in Fischer 344 rats through both its influence on MNCL proliferation via suppression of the GH:IGF-1 axis and its enhancement of host defenses against tumor cells. Since diet restriction has been shown to modulate tumor outcomes in a variety of experimental paradigms, opportunities are widespread for evaluating the roles of GH and IGF-1 in mediating these responses.

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INHIBITION OF LEUKEMIA BY DIET RESTRICTION


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